AD_____

Award Number: W81XWH-04-1-0457

TITLE: Role of Polyamine Oxidase (PAOH1/SMO) in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Allison M. Pledgie

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205-2196

REPORT DATE: April 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Annual Summary (22 Mar 2004 - 21 Mar 2005)

4. TITLE AND SUBTITLE
Role of Polyamine Oxidase (PAOH1/SMO) in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0167

6. AUTHOR(S)
Allison M. Pledgie

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205-2196

E-Mail: apledgie@jhsph.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Bis(ethyl)norspermine (BENSpm) is a spermine analogue that has shown antitumor activity in multiple human tumor models. This project evaluates the ability of BENSpm to induce the polyamine catabolic enzyme spermine oxidase, SMO(PAOh1), in human breast cancer cell lines and examined the role of its induction in the overall response of breast cancer cell lines to BENSpm treatment. BENSpm differentially induced SMO(PAOh1) mRNA and activity in several breast cancer cell lines. The greatest induction was seen in MDA-MB-231 cells, which exhibited a time- and dose- dependent induction of SMO(PAOh1) mRNA and activity with BENSpm treatment. Although SMO(PAOh1) was not induced in MCF-7 and T47D cells, BENSpm exposure inhibited cell growth, decreased intracellular polyamine levels, decreased ODC activity, and induced SSAT activity in all of the cell lines examined. Inhibition of SMO(PAOh1) activity with the polyamine oxidase inhibitor MDL 72,527 reduced the sensitivity of MDA-MB-231 and Hs578t cells to BENSpm but had no effect on the response of MCF-7 and T47D cells to exposure to BENSpm. These results indicate that the induction of SMO(PAOh1) may play a role in the response of specific breast cancer cell lines to BENSpm treatment.

14. SUBJECT TERMS
Polyamine oxidase, polyamine analogue, polyamine oxidase sílice variants

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The natural polyamines, spermine, spermidine, and their diamine precursor, putrescine, are ubiquitous polycationic bases that are required for normal cell growth and differentiation (1-4). Breast cancer tissues demonstrate aberrant regulation of the polyamine metabolic pathway, including increased intracellular polyamine levels and decreased activity of SMO(PAOh1), one of the key regulators of polyamine degradation (5, 6). SMO(PAOh1) oxidizes spermine to spermidine and results in the production of H_2O_2, a reactive oxygen species (7-9). Diminished SMO(PAOh1) activity in breast cancer may be associated with reduced levels of H_2O_2 and decreased cell growth inhibition and apoptosis, thereby contributing to breast cancer cell growth. Recently, the activity of SMO(PAOh1) was found to be differentially inducible in several human lung cancer cell lines following exposure to polyamine analogues (10, 11). The hypothesis underlying this project is that induction of SMO(PAOh1) activity by polyamine analogues will deplete intracellular polyamine levels, enhance H_2O_2 production, and lead to growth inhibition and/or activate apoptotic cell death in human breast cancer cell lines.

BODY

Specific Aim 1: To determine the levels of SMO(PAOh1) mRNA, protein expression, and activity in normal and malignant breast cell lines. The induction of SMO(PAOh1) mRNA with BENSpm was first examined by RT-PCR in seven breast cancer cell lines that represent a wide range of breast cancer phenotypes, as well as two immortalized, non-tumorigenic mammary epithelial cell lines. SMO(PAOh1) mRNA was detected at a very low level in each untreated cell line. After treatment with 10 μM BENSpm for 24 hours, a large induction of SMO(PAOh1) mRNA was observed in the two estrogen receptor (ER) negative cell lines, MDA-MB-231 and Hs578t cells, whereas no induction of SMO(PAOh1) mRNA was seen in the two ER positive cell lines, MCF-7 and T47D.

To further investigate the differential induction of SMO(PAOh1) in response to BENSpm treatment, MDA-MB-231 and MCF-7 cell lines, which exhibit a large and small induction of SMO(PAOh1) mRNA, respectively, were chosen for further study. Both cell lines were treated for 24 hours with a range (0 – 50 μM) of BENSpm concentrations. Treatment of MDA-MB-231 cells with BENSpm resulted in a dose-dependent increase in SMO(PAOh1) mRNA expression and enzyme activity whereas no significant change in either parameter was observed using up to 50 μM BENSpm in MCF-7 cells. SMO(PAOh1) mRNA was induced in MDA-MB-231 cells with concentrations up to 10 μM BENSpm, which is the concentration that maximal activity occurred with. This concentration was used to examine the time course of SMO(PAOh1) mRNA and activity induction in both cell lines with BENSpm (0 – 48 hours). Induction of SMO(PAOh1) mRNA in MDA-MB-231 cells began after six hours of treatment with BENSpm with maximal induction achieved by 24 hours. SMO(PAOh1) activity was induced after nine hours and increased through 48 hours of BENSpm treatment. In contrast, SMO(PAOh1) mRNA is minimally induced in BENSpm treated MCF-7 cells after 48 hours and no significant induction is seen in enzyme activity. RT-PCR and real time PCR studies

demonstrated that BENSpm induced the expression of each of the four previously described SMO(PAOh1) variants in MDA-MB-231 cells by about six-fold. Unfortunately, a SMO(PAOh1) antibody has not yet been developed for Western blotting.

Specific Aim 2: To determine the effects of multiple classes of polyamine analogues on cell growth and death, induction of SMO(PAOh1), related polyamine pathway enzyme activities, and intracellular polyamine levels MDA-MB-231 and MCF-7 cells were treated with 10 μM BENSpm for 24 hours and the effects on intracellular polyamine levels, ODC activity, and SSAT activity were measured. In BENSpm-treated MDA-MB-231 and MCF-7 cells, the levels of spermine, spermidine, and putrescine decreased by nearly 50% with a similar level of BENSpm accumulation in both cell lines. BENSpm treatment reduced ODC activity in MDA-MB-231 cells 8-fold and in MCF-7 cells 16-fold and induced SSAT activity 98-fold in MDA-MB-231 cells and 19-fold in MCF-7 cells.

The impact of the differential induction of SMO(PAOh1) by BENSpm on breast cancer cell growth was examined by treating MDA-MB-231, Hs578t, MCF-7 and T47D cells with 10 μM BENSpm for 96 hours and cell numbers were counted every 24 hours. Treatment of each cell line with BENSpm for 48 hours or longer significantly inhibited cell growth. FACS analysis showed no difference in cell cycle effects in BENSpm treated MDA-MB-231 and MCF-7 cells; both cell lines arrested in G1 after 48 hours of BENSpm treatment and remained in a G1 block through 96 hours. The effects of other classes of polyamine analogues on these parameters will be evaluated in the future.

A key question is whether BENSpm-induced SMO(PAOh1) activity plays a role in growth inhibition of MDA-MB-231 cells. To address this question, a pharmacologic approach was used. MDA-MB-231, Hs578t, MCF-7 and T47D cells were co-treated with 10 μM BENSpm and 25 μM MDL 72,527, an inhibitor that effectively inhibits both PAO and SMO(PAOh1), and cell growth assays were performed. Inhibition of oxidase activity with MDL 72,527 significantly reduced the sensitivity of MDA-MB-231 and Hs578t cells to BENSpm treatment while there was no change in the sensitivity of MCF-7 and T47D cells to BENSpm by co-treating with MDL 72,527 using this time course.

Since MDL 72,527 inhibits both SMO(PAOh1) and the acetyl-polyamine oxidase (PAO), an RNA interference strategy will be used to confirm that SMO(PAOh1) plays a specific role in BENSpm response in MDA-MB-231 cells. MDA-MB-231 and MCF-7 cells will be stably transfected with vectors that transcribed small interfering RNAs directed against exon 2 of the SMO(PAOh1) gene or a nonsense sequence and a stable population of cells will be selected by growth in G418.

Specific Aim 3: To determine the effects of SMO(PAOh1) over-expression on cell growth and death, related polyamine pathway enzyme activities, and intracellular polyamine levels. Future experiments will address the goals of this aim.

KEY RESEARCH ACCOMPLISHMENTS

- BENSpm differentially induces SMO(PAOh1) mRNA and activity in multiple human breast cancer cell lines.
- BENSpm induces SMO(PAOh1) mRNA and activity in a time- and dose-dependent manner in MDA-MB-231 cells.
- BENSpm induces the mRNA expression of each of the four identified SMO(PAOh1) splice variants 4-6 fold in MDA-MB-231 cells.
- BENSpm exposure inhibits cell growth, reduces intracellular polyamine levels, induces SSAT activity, and reduces ODC activity in multiple breast cancer cell lines regardless of SMO(PAOh1) induction.
- Co-treatment of MDA-MB-231 and Hs578t cells with BENSpm and MDL 72527, the polyamine oxidase inhibitor, significantly reduces their sensitivity to BENSpm but does not alter the sensitivity of MCF-7 and T47D cells to BENSpm.

REPORTABLE OUTCOMES

- *Abstracts:*
  - **Differential induction of human spermine oxidase mRNA and activity in human breast cancer cell lines.** Pledgie A, Huang Y, Hacker A, Woster PM, Casero RA, and Davidson NE. Proc AACR. Page 1224, 2004.
- *Presentations:*
  - **The role of spermine oxidase in human breast cancer.** Pledgie A. Breast Cancer Program Seminar Series, Department of Oncology, Johns Hopkins University, May 2004.

CONCLUSIONS

This study demonstrates that the recently cloned human spermine oxidase, SMO(PAOh1), is differentially inducible with BENSpm in several breast cancer cell lines. These results suggest that the induction of SMO(PAOh1) plays a role in the growth inhibitory response of MDA-MB-231 and Hs578t cells to BENSpm exposure. Inhibition of SMO(PAOh1) activity with MDL 72,527 can delay, but not completely inhibit, the response to BENSpm, suggesting that BENSpm activates multiple pathways of which SMO(PAOh1) is just one. Future work will involve stably knocking down the expression of SMO(PAOh1) using RNA interference in MDA-MB-231 and MCF-7 cells to confirm the results obtained through pharmacological inhibition of oxidase activity. The oxidation-sensitive fluorescent probe CM-H_2DCFDA will also be used to detect the production of H_2O_2 by induction of SMO(PAOh1) activity. Further understanding of the molecular targets of BENSpm will allow for the development of more cytotoxic agents for the treatment of breast cancer. Future work will be aimed at examining other targets of BENSpm that act in combination with SMO(PAOh1). This study has identified SSAT and ODC as other targets of BENSpm. While SMO(PAOh1) was differentially inducible in breast cancer cell lines, SSAT activity was induced and ODC activity was reduced in all breast cancer cell lines examined. It will be critical to examine the roles that other enzymes in the polyamine metabolic pathway, notably SSAT and ODC, play in response to BENSpm in breast cancer cell lines.

REFERENCES

1. Wallace HM, Fraser AV, and Hughes A. A perspective of polyamine metabolism. Biochem J. 376: 1-14, 2003.
2. Huang Y, Pledgie A, Casero RA, and Davidson NE. Molecular mechanisms of polyamine analogs in cancer cells. Anticancer Drugs. 16: 229-41, 2005.
3. Thomas T and Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and their applications. Cell Mol Life Sci. 58: 244-58, 2001.
4. Marton LJ and Pegg AE. Polyamines as targets for therapeutic intervention. Annu Rev Pharmacology Toxicol. 35: 55-91, 1995.
5. Kingsnorth AN and Wallace HM. Elevation of monoacetylated polyamines in human breast cancers. Eur J Cancer Clin Oncol. 21: 1057-62, 1985.
6. Kingsnorth AN, Wallace HM, Bundred NJ, and Nixon JM. Polyamines in breast cancer. Br J Surg. 71: 352-6, 1984.
7. Wang Y, Murray-Stewart T, Devereux W, Hacker A, Frydman B, Woster PM, and Casero RA. Properties of purified recombinant human polyamine oxidase, PAOh1/SMO. Biochem Biophys Res Commun. 304: 605-11, 2003.
8. Wang Y, Hacker A, Murray-Stewart T, Fleischer JG, Woster PM, and Casero RA. Induction of human spermine oxidase SMO(PAOh1) is regulated at the levels of new mRNA synthesis, mRNA stabilization, and newly synthesized protein. Biochem J, 2004.
9. Murray-Stewart T, Wang Y, Devereux W, and Casero RA. Cloning and characterization of multiple human polyamine oxidase splice variants that code for isoenzymes with different biochemical characteristics. Biochem J. 368: 673-7, 2002.
10. Wang Y, Devereux W, Woster PM, Stewart TM, Hacker A, and Casero RA. Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. Cancer Res. 61: 5370-3, 2001.
11. Devereux W, Wang Y, Stewart TM, Hacker A, Smith R, Frydman B, Valasinas AL, Reddy VK, Marton LJ, Ward TD, Woster PM, and Casero RA. Induction of the SMO(PAOh1) polyamine oxidase by polyamine analogues in human lung cancer cells. Cancer Chemother Pharmacol. 52: 383-90, 2003.